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Antihyperlipidemic and Antioxidant Activities of Flavonoid-Rich Extract of *Ziziphus lotus* (L.) Lam. Fruits

Noureddine Bencheikh [1], Mohamed Bouhrim [1], Ilyass Alami Merrouni [1], Salima Boutahiri [2,3], Loubna Kharchoufa [1], Mohamed Addi [4,*], Duangjai Tungmunnithum [5], Christophe Hano [6], Bruno Eto [3], Abdelkhaleq Legssyer [1] and Mostafa Elachouri [1]

1 Laboratory of Bioresources, Biotechnology, Ethnopharmacology, and Health, Faculty of Sciences, Mohammed First University, Oujda 60040, Morocco; bencheikh_noureddine1718@ump.ac.ma (N.B.); mohamed.bouhrim@gmail.com (M.B.); alami.ilyass.90@gmail.com (I.A.M.); l.kharchoufa@ump.ac.ma (L.K.); a.legssyer@ump.ac.ma (A.L.); elachourimostafa@gmail.com (M.E.)
2 Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Lille, 3, rue du Professeur Laguesse, B.P. 83, 59000 Lille, France; boutahiri.salima@gmail.com
3 Laboratoire TBC, Laboratory of Pharmacology, Pharmacokinetics and Clinical Pharmacy, Faculty of Pharmacy, University of Lille, 3, rue du Professeur Laguesse, B.P. 83, 59000 Lille, France; titisfrance@gmail.com
4 Laboratoire d'Amélioration des Productions Agricoles, Biotechnologie et Environnement, (LAPABE), Faculté des Sciences, Université Mohammed Premier, Oujda 60000, Morocco
5 Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand; duangjai.tun@mahidol.ac.th
6 Laboratoire de Biologie des Ligneux et des Grandes Cultures, INRAE USC1328, Campus Eure et Loir, Orleans University, 28000 Chartres, France; hano@univ-orleans.fr
* Correspondence: m.addi@ump.ac.ma

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Abstract: *Ziziphus lotus* (L.) Lam. (*Z. lotus*) is a medicinal plant species that is widely distributed throughout the Mediterranean basin. Moroccans traditionally use it to treat many illnesses thanks to its beneficial medicinal properties. The purpose of this study is to assess the anti-hyperlipidemic and antioxidant activities of a flavonoid-rich aqueous extract of *Z. lotus* fruits (ZLF). The 2-2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay and the β-carotene bleaching test were used to determine the antioxidant activity of ZLF. The anti-hyperlipidemic effect of the aqueous extract of ZLF (200 and 400 mg/kg) was evaluated in albino mice exposed to the chronic high-fat diet, based on lipid profile, blood sugar, and changes in growth performance. The results showed that the aqueous extract of ZLF rich in flavonoids (2242.89 $\pm$ 25 µg QE (quercitin equivalent)/mg), and has a considerable antioxidant power against DPPH radicals (IC_{50} = 116 $\pm$ 0.02 µg/mL) and β-carotene oxidation. The aqueous extract of ZLF also showed a significant anti-hyperlipidemic effect by improving abnormal changes in lipid profile and blood glucose levels in albino mice exposed to a chronic high-fat diet. Our findings suggest that the anti-hyperlipidemic activities of ZLF aqueous extract are correlated with its flavonoid content and antioxidant activity. Therefore, the aqueous extract of ZLF could be an essential therapeutic candidate for hyperlipidemia patients, thanks to its richness in bioactive molecules.

Keywords: *Ziziphus lotus* L. (Lam.); flavonoids; antioxidant; anti-hyperlipidemia

1. Introduction

Medicinal plants have been known for thousands of years and are widely appreciated as a rich source of therapeutic agents to prevent and cure various ailments. According to The Plant List (2013), the genus *Ziziphus* encompasses 58 accepted plant species and are widely used for their therapeutic properties. Their plants are small trees were thorny shrubs, which are generally distributed in the subtropical and warm temperate regions of the world [1]. Among the most important plants of this genus, *Z. lotus* known as Jujube belongs to the angiosperm *Rhamnaceae* family. It is a Mediterranean medicinal plant that grows in Africa and some European countries such as Spain, Cyprus, Greece, and

several Asian countries, such as China, Iran, and South Korea [2,3]. In Morocco, *Z. lotus* is popularly known as "Sedra," and its fruits as "Nbeg", and has widely distributed in arid and semi-arid areas [4]. The fruits of this plant have been used for various purposes such as nutrition, health, and thus used in cosmetics in the form of a mixture. Different parts of this plant have been traditionally used in Morocco to treat various health problems, such as cardiovascular diseases, urinary tract infections, liver disorders, gastrointestinal problems, insomnia, diarrhea, skin infections, and diabetes [4–6]. It has been reported that extracts of the fruits of *Z. lotus* have shown considerable pharmacological properties, such as antioxidant [7], the lithophytic [4], antidiabetic, dermatoprotective [7], hepatoprotective effects [8], antispasmodic [9], anti-inflammatory, analgesic [10], antiulcerogenic [11], and gastroprotective effect [12]. Besides, this plant is well known for its safety [13]. A recently published phytochemical study showed that the presence of a total of 20 phenolic and flavonoid compounds in the alcoholic extracts of the fruits of *Z. lotus* including (−)-catechin 3-*O*-gallate, quercetin derivatives (such as quercetin rhamnosyl-rhamnosyl glucoside, quercetin di-glucoside, and quercetin rhamnoside glucoside), or eriodictyol derivatives [14]. Similarly, in the aqueous extract of the fruits of *Z. lotus*, Marmouzi and its collaborators (2019) identify twenty phenolic compounds, including catechin and rutin [7].

Hyperlipidemia and oxidative stress have become major health concerns in recent years. These parameters are known to be the main risk factors contributing to the development and progression of atherosclerosis and associated cardiovascular and cerebrovascular diseases [15]. As a result, interest has recently increased in the use of new natural antioxidants, mainly of plant origin [16]. It is well known that flavonoids and phenolic compounds of vegetable origin have multiple pharmacological properties, particularly the antioxidant and anti-hyperlipidemic effects [17–19]. To our best knowledge, no study has been done to evaluate the antioxidant and anti-hyperlipidemia effect of the fruits of *Z. lotus* of the Eastern Moroccan region. In this respect, the objective of our study is to assess the anti-hyperlipidemia effect of the aqueous extract of ZLF rich in flavonoids in mice fed a high-fat diet and to investigate the mechanism of this effect. Antioxidant activity was also evaluated in vitro cell-free assays.

2. Materials and Methods

2.1. Chemicals

The DPPH, cholesterol, linoleic acid, aluminum chloride ($AlCl_3$), β-carotene, butylated hydroxytoluene (BHT), tween-80, methanol, chloroform, Folin–Ciocalteu reagent, gallic acid, ascorbic acid, and quercetin were purchased from Sigma-Aldrich (Steinheim, Germany). All other reagents and chemicals used are of analytical quality.

2.2. ZLF Aqueous Extract Preparation

The fruits of the plant were harvested in the region of Oujda (Morocco) in October 2020 (Figure 1). The collected fruits were identified and authenticated by the botanist Mohammed Fennane from the scientific institute of the University Mohammed V. The specimen was prepared and deposited in the Herbarium of the University Mohammed first Oujda-Morocco "HUMPOM" under the voucher number HUMPOM428. The ZLF extract was prepared based on the traditional technique used by the Moroccan population. Indeed, the ZLF was transformed into powder, and the aqueous extract was then prepared by adding 100 g of ZLF powder to 2000 mL of boiled distilled water (70 °C), and the mixture was stirred for 20 min. The mixture was filtered, and the filtrate evaporated to remove the water and obtain the extract as a powder.

(**A**) (**B**)

Figure 1. (**A**) *Z. lotus* and its habitat; (**B**) Fruits of *Z. lotus*.

2.3. Determination of Total Flavonoid and Phenolic Contents

The quantification of total flavonoids was performed using the method described by Kim et al. (2003) with slight modification [20]. Indeed, the reaction mixture contains 200 µL of the aqueous ZLF's extracts (1000 µg/mL), 1000 µL of distilled water, and 50 µL of $NaNO_2$ (5%). After 6 min of homogenization, we added 120 µL of $AlCl_3$ (10%), incubated it for 5 min, and then added 400 µL of NaOH (1 M) and 230 µL of distilled water. Finally, we measured the absorbance of the mixture spectrophotometrically at a wavelength of 510 nm, using methanol as a blank. Data were expressed as µg QE/mg of dry matter.

The quantification of the total amount of phenols in the aqueous extract of ZLF was performed using the Folin–Ciocalteu method [21]. Briefly, 200 µL of the ZLF's aqueous extract (1000 µg/mL) were mixed with 1000 µL of Folin–Ciocalteu reagent and 800 µL of Na_2CO_3 (75 g/L). The reaction mixture was vortexed and incubated at room temperature for one hour. Then, the absorbance was measured by spectrophotometer at a wavelength of 765 nm against the blank (methanol). The results were expressed in µg EAG (Equivalent Acid Gallic)/mg of dry matter.

2.4. Determination of the Antioxidant Activity

2.4.1. DPPH Free Radical-Scavenging Activity Assay

The DPPH radical scavenging assay was done, following the method described by [22], with some modifications. Indeed, increasing concentrations of the aqueous ZLF extract (6, 12, 25, 50, 100, 200, and 400 µg/mL) were prepared in the test tubes, then taken 0.1 mL from each tube and mixed with 2.5 mL of the ethanolic solution of the DPPH (4%). The mixtures were then incubated in the dark at room temperature for 30 min. After incubation, the absorbance of the mixtures was immediately measured by spectrophotometry at a wavelength of 517 nm. Ascorbic acid was used as a reference (a standard antioxidant). All tests were done in triplicate. The trapping activity of our extract was calculated using the following formula:

$$\text{DPPH trapping effect } (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

A_0 is the absorbance of the control reaction, and A_1 is the absorbance of all of the extract samples and standards.

2.4.2. β-Carotene Bleaching Assay

The antioxidant activity of ZLF extract was evaluated following the beta-carotene bleaching protocol described by Sun and Ho [23]. Indeed, a solution contains 2 mg of

β-carotene, and 10 mL of chloroform were mixed with 20 mg of linoleic acid and 200 mg of Tween-80. After removing the chloroform mixture by rotavapor at 40 °C, 100 mL of distilled water was added to the flask under vigorous agitation. Then, 0.2 mL of this mixture was added to the individual test tubes containing the sample solution, and then the tubes were incubated at 50 °C in a water bath under continuous agitation for 2 h. Absorbance was measured at 0 and 2 h of incubation at a wavelength of 470 nm. BHT was used as a standard and distilled water was used as negative control (NC). The measurements were repeated three times to minimize the error. The percentage of oxidized β-carbon was calculated using the following formula:

$$(\%) \text{ oxidized } \beta - \text{ carotene} = \frac{Ai(to) - Af(2h)}{\text{Ai(to)}} \times 100$$

Ai: absorbance in t_o, and *Af*: absorbance after incubation (2 h).

2.5. Anti-Hyperlipidemic Activity

2.5.1. Animals and Housing

Twenty-four male Swiss albino mice (19 weeks old) weighing between 20 and 30 g were used in this study. All animals were kept in plastic cages with free access to food and water. The animals were adapted to standard pet shop conditions (23 °C, 12 h dark/12 h bright) 15 days before treatment. The mice were cared for in compliance according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the Faculty of Sciences, Oujda, Morocco (01/20-LBBEH-04 and 09/01/2020).

2.5.2. Preparation of High-Fat Diet

The high-fat diet was prepared according to the protocol described by Harnafi et al. (2009) [24]; this diet consisted of the regular chow diet (Society SONABETAIL, Oujda, Morocco), cholesterol 2%, fat 16%, and deoxycholic acid 0.2%.

2.5.3. Experimental Protocol Design

After two weeks of acclimatization, the mice were divided into four equal groups (n = 6) and treated as follows:

- A standard control group (NCG) received only distilled water at 10 mL/kg body weight.
- The Hyperlipidemic Control Group (HCG) freely received the high-fat diet and received distilled water daily (10 mL/kg).
- Two treated groups received 200 or 400 mg/kg of ZLF aqueous extract and then a high-fat diet for 30 days of the treatment.

At the end of the treatment, all mice were anesthetized by a light ethyl ether inhalation and sacrificed. The blood samples were then taken from their retroorbital sinus into heparinized tubes and centrifuged at 3000 rpm for 10 min, at 4 °C, to separate the plasma. The separated plasma was stored at −20 °C for further evaluation.

2.5.4. Biochemical Analysis

The biochemical parameters have been measured in plasma. The commercial kits measured high-density lipoprotein cholesterol (HDL-c), triglycerides (TG), total cholesterol (TC), and blood glucose levels. All of the tests were performed with the COBAS INTEGRA® 400-Plus analyzer (Roche Diagnostics, Meylan, France).

2.5.5. The Atherogenic Index Calculation

The atherogenic index was calculated by the following formula:

AI = (Total cholesterol) − (HDL-C)/HDL-C, and in the LDL-C/HDL C, the ratio was calculated as the ratio of plasma LDL-C to HDL-C levels.

2.6. Statistical Analysis

Data were expressed as means ± standard error of the mean (SEM). Statistics and graphical representation were performed using Graph Pad Prism 5 software, San Diego, CA, USA, by regular one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. The difference was significant if $p < 0.05$, moderately significant if $p < 0.01$, and highly significant if $p < 0.001$.

3. Results and Discussion

3.1. Total Phenolic and Flavonoids Contents

The quantification of the total phenolic and flavonoids contents was showed that the ZLF aqueous extract is rich in flavonoids (2242.89 ± 25 µg QE/mg) and to a lesser extent in total phenolic (278 ± 12 µg AGE/mg). These results confirm the results of a previously published study on ZLF from the same country, which showed that the aqueous extract of ZLF rich in total phenolic (285.19 µg EAG/MG of dry extracts) compared to the n-hexane extract of the fruits of this plant [4]. In addition, an analysis by HPLC-DAD-ESI/MS shows that aqueous ZLF extracts are rich in phenolic compounds such as sinapic acid, p-Hydroxybenzoic acid, p-coumaric acid, p-coumaroyl glucose, benzoic acid, cinnamic acid derivative, galloyl shikimic acid, (−)-catechin 3-O-gallate, and quercetin [14]. These compounds, thanks to their interesting antioxidant power, have a wide range of pharmacological activities, including anti-hyperlipidemic activity [25]. As components of the human diet, these phenolic compounds are able to act as antioxidants directly to influence the production or functioning of other antioxidant compounds in the body. These compounds can improve health, as they are able to act in many functional roles, acting as free radical trappers, singlet and triplet oxygen extinguishers, and peroxide decomposers [26,27].

3.2. Antioxidant Activity of ZLF Aqueous Extract

Antioxidants and/or reactive oxygen species (ROS) scavengers, such as flavonoids and phenolics, have been shown to slow the progression of atherosclerosis in a variety of hyperlipidemic models [28–30]. As a result, the antioxidant capacity of ZLF aqueous extract was assessed using two distinct assays (i.e., DPPH free radical scavenging assay and β-carotene bleaching test) the first step in our research.

3.2.1. DPPH Free Radical Scavenging Activity

The antioxidant effect of ZLF aqueous extract was evaluated on a concentration series, and the results of the DPPH trapping activity are presented in Figure 2. The aqueous extract of ZLF exhibited inhibitory activity against the free radical DPPH in a dose-dependent manner. The half-maximum inhibitory concentration of the aqueous ZLF extract was IC_{50} = 116 ± 0.02 µg/mL, and was higher than that of ascorbic acid who has an IC_{50} = 27.02 ± 0.02 µg/mL. These results showed that the aqueous ZLF extract has considerable antioxidant activity against DPPH free radicals. This antioxidant activity of our extract is in agreement with this found by Marmouzi et al. (2019) in the aqueous extract of ZLF from the region of Sidi Sliman (Morocco) [7]. Moreover, ZLF from different regions of Algeria was showed a higher potential for free radical trapping DPPH [31]. In our work, we have shown that the aqueous extract of this plant is rich in polyphenol and flavonoids. However, phenolic and flavonoids compounds are known to have a high antioxidant effect against various reactive oxygen and nitrogen species [32]. Therefore, the antioxidant activity of our extract could be due to its high content of polyphenols and flavonoids.

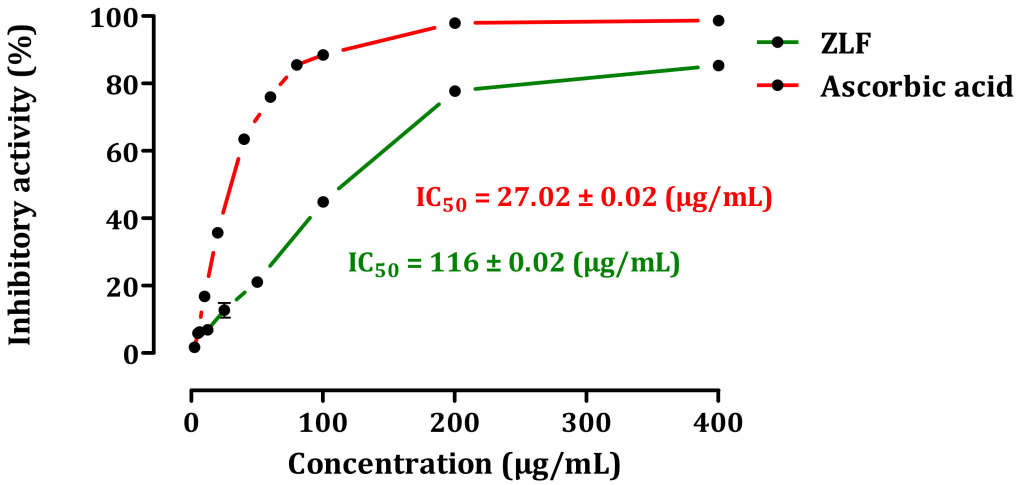

Figure 2. DPPH Free radical scavenging activity of ZLF extract and ascorbic acid.

3.2.2. β-Carotene Bleaching Test

The results of β-carotene bleaching test of aqueous ZLF extract are shown in Figure 3. The β-carotene bleaching test of ZLF extract showed considerable inhibition in a concentration way depend. Indeed, the oxidation percentages of β-carotene in the presence of the aqueous extract of ZLF are 42.24%; 31.68%; 26.92%, and 21.11%, respectively, for the concentrations 12.5, 25, 50, and 100 µg/mL, and those of BHT were 3.50%, 1.85%, 1.02%, and 0.62%, respectively, for the concentrations 12.5, 25, 50, and 100 µg/mL. This inhibition of β-carotene oxidation of the aqueous ZLF extract may be due to the high levels of polyphenols and flavonoids present in this extract.

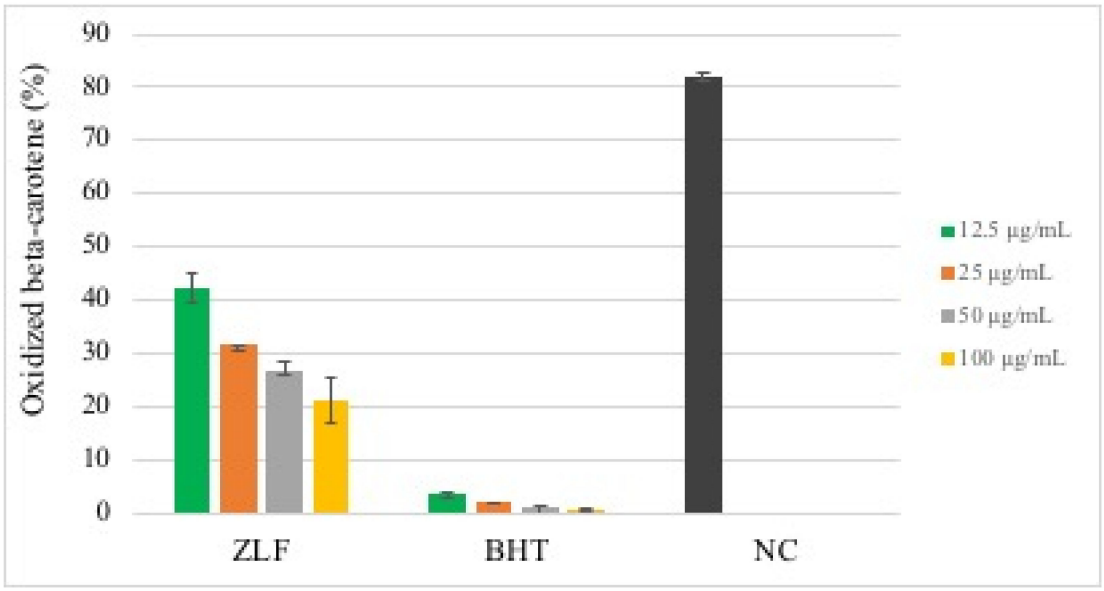

Figure 3. Effect of aqueous ZLF extract on β-carotene oxidative bleaching, NC: negative control; butylated hydroxytoluene (BHT).

3.3. Effect of the ZLF Aqueous Extract on High-Fat Diet-Induced Lipid Metabolism Disturbance in Mice

3.3.1. Bodyweight and Relative Weight of Organs

The experiment measured bodyweight gain, relative kidney, and liver weight in all mice (Table 1). Mice fed a high-fat diet show a slight, non-significant increase in body weight gain and relative kidney and liver weight compared to normolipidemic mice (NCG).

This non-significant increase could be explained by the short duration of the high-fat diet administration, which is insufficient to induce a significant increase in growth performance. It is pretty standard that a regular diet high in fat caused obesity in mice, and this has been well demonstrated by several studies [33,34]. The daily administration of ZLF aqueous extract for four weeks of treatment mitigated the increase in body weight that was not significant, the relative weight of the kidneys, and the relative weight of the liver in mice fed a high-fat diet compared to NCG mice. These results are consistent with several previously published studies to assess the anti-hyperlipidemia effects of plant extracts [16].

Table 1. Bodyweight and Relative Weight of Organs.

Groups	Weight Gain (g)	Relative Kidney to Body Weight (g/100 g of bw)	Relative Liver to Body Weight (g/100 g of bw)
NCG	5.61 ± 0.73	0.67 ± 0.16	3.81 ± 0.92
HCG	7.39 ± 0.21	0.79 ± 0.15	4.51 ± 0.83
ZLF (200 mg/Kg)	6.11 ± 1.01	0.72 ± 0.11	4.13 ± 0.71
ZLF (400 mg/Kg)	5.31 ± 0.92	0.69 ± 0.09	4.01 ± 0.25

3.3.2. The Effect of ZLF on Triglycerides, Total Cholesterol, and HDL-Cholesterol

The effect of aqueous ZLF extract on the plasma level of triglyceride, total cholesterol, and HDL-cholesterol in mice exposed to a chronic high-fat diet was presented in Figure 4. Indeed, a significant increase in triglycerides ($p < 0.001$), and total cholesterol ($p < 0.001$), and a non-significant decrease in HDL cholesterol were observed for the mice of HCG compared to the mice of NCG. However, the daily intake of ZLF aqueous extract at 200 and 400 mg/kg during four weeks of treatment significantly harmonized the increase in triglyceride and total cholesterol for the mice of HCG compared to mice of NCG. This suggests that the hypocholesterolemic activity of the aqueous ZLF extract may be due to the redistribution of plasma LDL-cholesterol to the liver and extra-hepatic tissues via these receptors at the level of hepatocytes, intended for elimination in the form of bile acids. In addition, to avoid cholesterol deposition in blood vessels, the abundance of cholesterol in peripheral tissues must be returned to the liver via HDL [35]. Indeed, the hypocholesterolemic property of the aqueous ZLF extract has been associated with a rise in plasma levels of HDL-cholesterol, leading to the elimination of cholesterol in the form of bile acids. This result is in harmony with previous work evaluating the hypolipidemic effect of plant extracts [36].

On the other hand, it has been recently reported that triglycerides play a crucial role in maintaining normal lipid metabolism by regulating lipoprotein interactions [25]. In our results, administration of the aqueous ZLF extract reduced plasma triglyceride levels in mice exposed to a chronic fat-rich diet. This result shows that our extract may restore the catabolism of triglycerides in mice with a high-fat diet. This effect may be due to the improved elimination of triglyceride-rich lipoproteins through the improved activity of lipase lipoproteins and improved absorption of triglycerides transported in VLDL by peripheral organs [37]. Another mechanism may be related to the regulation of intestinal uptake of TG mediated by pancreatic lipase that catalyzes the hydrolysis of triglycerides into mono- and diglycerides, which are captured by intestinal cells [38]. Although the direct involvement of TG in atherosclerosis is not yet confirmed, it has been reported that reducing serum TG levels is effective in preventing hyperlipidemia and treating heart disease [39].

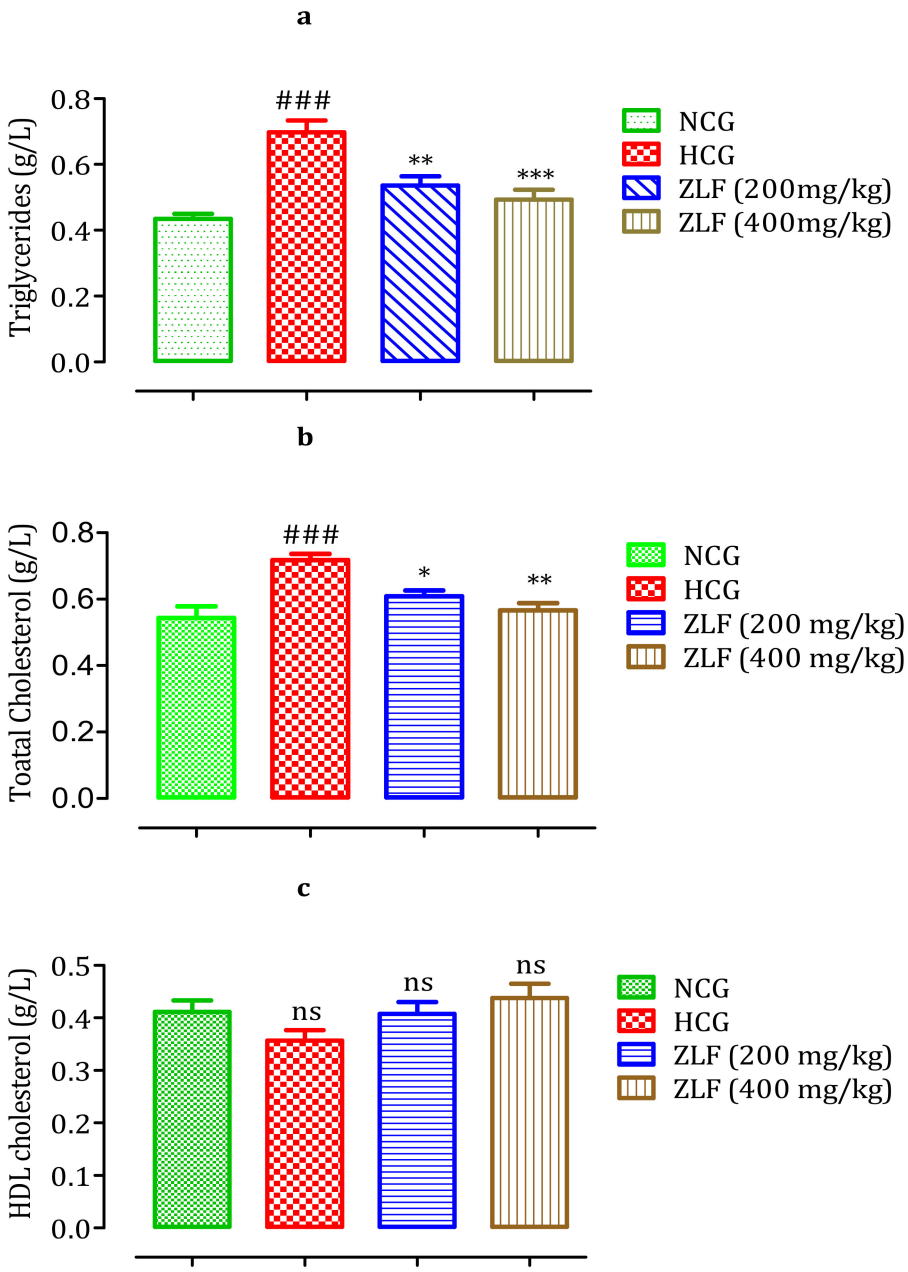

Figure 4. Effect of the aqueous ZLF extract on serum Triglycerides (**a**), Total cholesterol (**b**), and HDL-cholesterol (**c**) in mice exposed to HFD. Data are mean ± SEM, $n = 6$. $^{###}$ $p \leq 0.001$ versus NCG. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus HFD group. ns: not significant.

This could be explained by the fact that the aqueous ZLF extract stimulated the lipolytic activity of plasma lipoprotein lipase, as assumed by other users of different plants [40].

The anti-hyperlipidemia effects observed in this study can be attributed to the high concentration of polyphenols and flavonoids in the ZLF extract. Indeed, it has recently been reported that ZLF contains a large amount of catechin, which can form complexes with lipids and lipolytic enzymes, thus interfering with the emulsification of lipids, hydrolysis, micellar solubilization, and subsequent absorption [41]. Also, maybe by capturing free radicals to stop the chain reaction of lipid oxidation and preserving paraoxonase activity associated with HDL by chelating pro-oxidizing metal ions, thereby preventing LDL oxidation [42]. In this study, the results showed that ZLF is rich in several secondary

metabolites, including polyphenols and flavonoids. These substances have important anti-cholesterolemic and anti-hypertriglyceridic activities [31].

3.3.3. The Effect of ZLF on HDL Cholesterol/Total Cholesterol, and Atherogenic Index

The ratio of atherogenic index and total cholesterol/HDL-cholesterol was calculated in all mice in the experiment (Figure 5). Taking a chronic high-fat diet in mice induced a significant increase in the atherogenic index ($p < 0.001$) and in the total cholesterol/HDL-cholesterol ratio ($p < 0.001$) compared to NCG. However, daily administration of the aqueous ZLF extract at 200 and 400 mg/kg doses significantly suppressed the high values caused by the high-fat diet, which has almost returned to the base range. The reduction was significant for 200 and 400 mg/kg ($p < 0.05$ and $p < 0.01$, respectively) for the atherogenic index, and the reduction in total cholesterol/HDL-cholesterol ratio was significant ($p < 0.001$) at doses 200 and 400 mg/kg body weight compared to HCG mice. Indeed, a lower total cholesterol/HDL–cholesterol ratio helped prevent atherosclerosis as it was directly related to cardiovascular disease [43]. The ZLF extract significantly decreased the total cholesterol/HDL–cholesterol ratio in fat-overloaded mice, confirming that this extract has a beneficial effect on lipid profile regulation and ultimately prevents cardiovascular disease. This is consistent with other work examining the anti-hyperlipidemia effects of plant extracts [34,35,44,45].

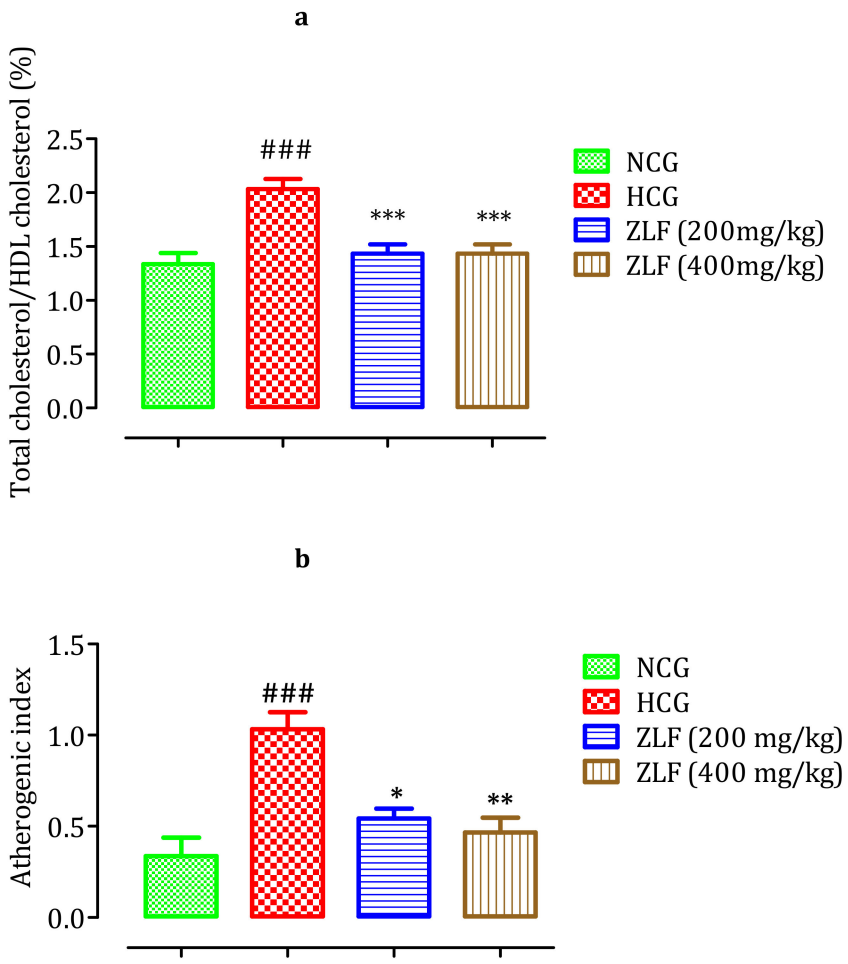

Figure 5. Effect of the aqueous ZLF extract on the ratio of total cholesterol/HDL-cholesterol (**a**) and Atherogenic index (**b**) in mice exposed to HFD. Data are mean ± SEM, $n = 6$. $^{###}$ $p \leq 0.001$ versus NCG. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus HFD group. ns: not significant.

3.3.4. The Effect of ZLF on Plasma Blood Sugar Levels

The effect of the ZLF aqueous extract on plasma glucose levels in high-fat diet mice was evaluated in all mice in the experiment (Figure 6). Mice exposed to a high-fat diet significantly increased ($p < 0.001$) in blood sugar compared to NCG mice. The hyperlipidemia is associated with hyperglycemia in approximately eightieth of patients. Hypertriglyceridemia which results from increased LDL caused by glycosylation lead to the hyperlipidemia [46]. However, administration of the ZLF aqueous extract at doses 200 and 400 mg/kg significantly ($p < 0.05$ and $p < 0.001$, respectively) maintained average blood glucose values in HCG mice compared to NCG mice. It is well known that abnormal lipid metabolism is one of the causes of insulin resistance disease in fat-overloaded mice [47]. Daily administration of aqueous ZLF extract to mice exposed to the chronic high-fat diet significantly reduced the increase in serum glucose compared to HCG mice. A study conducted by Marmouzi et al. (2019) showed that the aqueous extract of ZLF has an antidiabetic effect via in vitro inhibition of α-amylase and α-glucosidase, in which it demonstrated a higher effect of the extract compared to the standard drug acarbose [7]. In addition, a previous study revealed the antidiabetic effects of ZLF root and leaf extract on animal models, and this effect was attributed to vitamin A [48]. The results of our study showed that the fruits of *Z. lotus* are rich in several secondary metabolites, including polyphenols and flavonoids. These substances had significant anti-hyperglycemic activity [31].

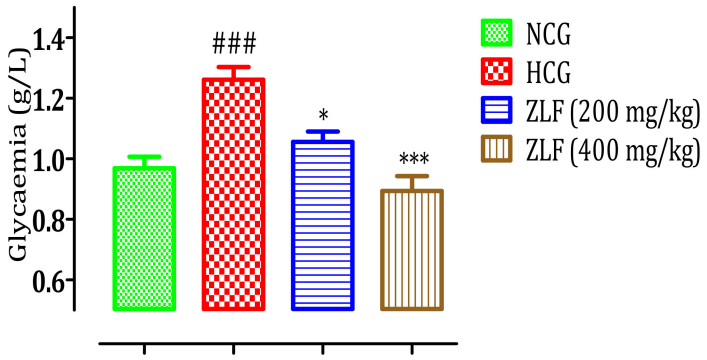

Figure 6. Effect of the aqueous ZLF extract on blood glucose level in mice exposed to HFD. Data are mean ± SEM, $n = 6$. ### $p \leq 0.001$ versus NCG. * $p < 0.05$, *** $p < 0.001$ versus HFD group.

4. Conclusions

In conclusion, the present results suggest that the aqueous extract of ZLF exerts considerable antioxidant activities as well as a powerful anti-hyperlipidemia and anti-atherogenic effects in the mice model. This effect could be due to their phenolic compounds, which could be set to react to trap free radicals to prevent lipid peroxidation. Therefore, the aqueous extract of ZLF could be exploited as a dietary supplement for subjects with hyperlipidemia.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All the data supporting the findings of this study are included in this article.

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Conflicts of Interest: The authors declare no conflict of interest.

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